PB85-212397

# Behavioral and Morphological Changes, in Fathead Minnow 'Pimephales promelas' as Diagnostic Endpoints for Screening Chemicals According to Mode of Action

(U.S.) Environmental Research Lab.-Duluth, MN

Jun 85

U.S. Department of Commerce
National Technical Information Service
NTIS

EPA/600/D-85/118
June 1985
PB85-212397

# Behavioral and Morphological Changes, in Fathead Minnow Pimephales promelas as Diagnostic Endpoints for Screening Chemicals According to Mode of Action

R. A. Drummond, C. L. Russom, D. L. Geiger[a], and D. L. DeFoe

U.S. Environmental Protection Agency
Environmental Research Laboratory-Duluth
6201 Congdon Boulevard
Duluth, Minnesota 55804

[a]Center for Lake Superior Environmental Studies
University of Wisconsin-Superior
Superior, Wisconsin 55880

REPRODUCED BY
NATIONAL TECHNICAL
INFORMATION SERVICE
U.S. DEPARTMENT OF COMMERCE
SPRINGFIELD, VA. 22161

**TECHNICAL REPORT DATA**
*(Please read Instructions on the reverse before completing)*

| 1. REPORT NO. | 2. | 3. RECIPIENT'S ACCESSION NO. |
|---|---|---|
| EPA/600/D-85/118 | | PB85 212397 /AS |

| 4. TITLE AND SUBTITLE | 5. REPORT DATE |
|---|---|
| Behavioral and Morphological Changes, in Fathead Minnow Pimephales promelas as Diagnostic Endpoints for Screening Chemicals According to Mode of Action | June 1985 |
| | 6. PERFORMING ORGANIZATION CODE |
| 7. AUTHOR(S) | 8. PERFORMING ORGANIZATION REPORT NO. |
| R.A. Drummond, C.L. Russon, D.L. Geiger, and D.L. DeFoe | |
| 9. PERFORMING ORGANIZATION NAME AND ADDRESS | 10. PROGRAM ELEMENT NO. |
| U.S. Environmental Protection Agency<br>Environmental Research Laboratory-Duluth<br>6201 Congdon Boulevard<br>Duluth, MN 55804 | 11. CONTRACT/GRANT NO. |
| 12. SPONSORING AGENCY NAME AND ADDRESS | 13. TYPE OF REPORT AND PERIOD COVERED |
| Environmental Research Laboratory<br>Office of Research and Development<br>U.S. Environmental Protection Agency<br>Duluth, MN 55804 | 14. SPONSORING AGENCY CODE<br>EPA-600/03 |

15. SUPPLEMENTARY NOTES

ASTM publication

16. ABSTRACT

Acute (96 hr) bioassays were conducted using 30-day old fathead minnows (Pimephales promelas). A total of 139 tests were performed under flow-through conditions at a temperature of 25 ± 1.0 C using Lake Superior water. A checklist was developed for systematically recording visual observations of behavioral and morphological changes. The checklist contained 40 different code items representing 10 general categories such as locomotor activity. Loss of schooling behavior appeared to be the most sensitive indicator of general stress. Although deviate changes in behavior were found to be sensitive indicators of stress, they cannot be used solely to classify chemicals according to mode of action. Gross morphological changes are equally important. Development of a model, based on behavioral and morphological data, appears possible for screening and differentiating chemicals according to toxic action.

17. KEY WORDS AND DOCUMENT ANALYSIS

| a. DESCRIPTORS | b. IDENTIFIERS/OPEN ENDED TERMS | c. COSATI Field/Group |
|---|---|---|
| | | |

| 18. DISTRIBUTION STATEMENT | 19. SECURITY CLASS (This Report) | 21. NO. OF PAGES |
|---|---|---|
| Release to public | Unclassified | 38 |
| | 20. SECURITY CLASS (This page) | 22. PRICE |
| | Unclassified | |

EPA Form 2220-1 (Rev. 4-77) PREVIOUS EDITION IS OBSOLETE

NOTICE

This document has been reviewed in accordance with U.S. Environmental Protection Agency policy and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

ABSTRACT: Acute (96 hr) bioassays were conducted using 30-day old fathead minnows (Pimephales promelas). A total of 139 tests were performed under flow-through conditions at a temperature of 25 ± 1.0 C using Lake Superior water. A checklist was developed for systematically recording visual observations of behavioral and morphological changes. The checklist contained 40 different code items representing 10 general categories such as locomotor activity. Loss of schooling behavior appeared to be the most sensitive indicator of general stress. Although deviate changes in behavior were found to be sensitive indictors of stress, they cannot be used solely to classify chemicals according to mode of action. Gross morphological changes are equally important. Development of a model, based on behavioral and morphological data, appears possible for screening and differentiating chemicals according to toxic action.

KEY WORDS: Freshwater fishes, minnows, bioassay, toxicology, behavior, responses, correlations, research.

INTRODUCTION

The main purpose of this study was to determine if various chemicals can be classified into groups using behavioral and morphological changes as indices [1]. Any scheme of classification can be used as long as it has meaning to those using the assigned categories [2]. Chemists place various chemicals into groups based on chemical structure. Physiologists and pharmacologists might label these same chemicals differently. The scope of this research involved development of a fish behavior checklist and establishment of a set of behavioral/morphological symptoms, for fathead minnows Pimephales promelas [3], specifically designed to separate individual chemicals into groups according to mode of action (MOA). It was assumed that if certain chemicals all cause fish to respond in a similar manner, then this set of symptoms can be used to classify these chemicals into a particular group [4,5,6,7].

Four better known modes of action were selected for in-depth study. They were anesthesia (narcosis) [8,9,10], uncouplers [11,12], acetylcholinesterase inhibitors [13,14] and skin irritants [15]. Two chemicals from each group, known in the literature to cause characteristic toxic symptoms, were chosen to represent these four modes. The eight chemicals served as a baseline for evaluating other chemicals of known and unknown MOA. These same eight chemicals are also being tested for their impact on fish respiration, uptake and depuration efficiencies, biochemical processes and cellular-organ damage. A comprehensive model for predicting MOA is anticipated upon completion of all studies. The present study is limited to behavioral and morphological changes as possible predictive endpoints.

Rapid screening techniques with predictive capabilities are needed to classify and single out those chemicals which might require further testing

[16,17,18]. One approach involves development of a quantitative structure-activity relationship for computing chemical toxicity. A model has been developed for those chemicals that kill by narcosis [19]. Not all chemicals fit the narcosis model and it was suspected that these outliers kill by some mode of action other than narcosis. Methods for discriminating between mechanisms are needed in order to predict lethal modes of action.

Fathead minnows were selected for study as they are ubiquitous and widely used in both acute and chronic toxicity tests. To date, there is little background data on their general behavior. Consequently their use in behavioral tests has been limited [3]. This study will help fill this gap.

## METHODS AND MATERIALS

### Test Conditions

Test apparatus--The continuous flow diluter used was a slightly modified version of Benoit's mini-diluter system [20] with the flow splitter cell going to only one tank (no duplicate tanks). Two flow rates were used: 10 ml/min and 25 ml/min per tank. The low flow system filled 2 L capacity tanks to a 1 L level. The dimensions were 22 x 10.7 x 4.5 cm. The high flow diluter filled tanks to a 2 L volume with a dimension of 22 x 10.7 x 9 cm. The high flow had 18 volume additions per day and the low flow had 14.4 volume additions per day. Both exposure systems had 6 tanks; 5 concentrations and a control, with a .65 dilution factor between concentrations. The low flow diluter was used primarily for testing chemicals that were expensive or in short supply, showed low toxicity, or had a low solubility rate.

Temperatures in the diluters were maintained at 25 $\pm$ 1.0 C. A 16 hr light and 8 hr dark photoperiod was maintained.

Dilution water and chemistries--Water used for testing and for toxicant stock dilutions was Lake Superior water which was filtered through sand and 5 $\mu$ cotton rope water filters. Water was preheated and aerated to prevent supersaturation of gases. All chemicals were added to the water without the aide of solvents or carriers of any kind. Physical-chemical analyses were performed using appropriate analytical procedures [21].

Test organism--Fathead minnows (*Pimephales promelas*) 28-34 days old were used in the flow-through tests. The fatheads were cultured and reared at the Environmental Research Laboratory in Duluth, Minnesota. Fish were not fed 24

---

Mention of brand names does not imply endorsement by the U.S. Environmental Protection Agency.

hr prior to or during the test. Twenty fish were tested at each concentration. Dead fish were removed daily during the exposures.

Video test area--An isolated test area was set-up for observing and documenting changes in behavior caused by the eight chemicals of known mode of action (MOA). These tests were in addition to the regular 96 hr acute bioassays. Briefly, this area consisted of 6 L tanks, each having a mirror positioned overhead at a 45 degree angle. The bottom of each tank was gridded into nine squares for monitoring horizontal fish dispersion. Rear walls were gridded into 'thirds' to assess relative vertical location. Additional 2 L tanks were set-up for making close-up recordings having maximum depth-of-field. A 1.27 cm (0.5 in) color video camera equipped with a macro-zoom lens was positioned in front of this test area. A VHS recorder, time-date generator and color monitor completed the video system.

Fish Behavior Checklist

Approach--Because of the large number (139) of test chemicals, construction of a toxicological "ethogram" [22] for each chemical was not attempted. Recording on-set, duration, intensity and sequence of behavioral symptoms for individual fish was impractical if not impossible. Our approach was to record mutually exclusive events [23,24] as opposed to using a graded or intensity of response scale. Since the data was systematically [25] collected over time, an overall measure of intensity, duration, etc., could be made for a group of fish exposed to a chemical. No attempt was made to delineate the numbers of fish responding. Behavioral and morphological aberrations were recorded whenever 10% or more of the fish in a given exposure tank appeared stressed. Behavioral activities of controls or "normal" fish were not recorded.

Checklist format--Behavioral and morphological abnormalities were recorded on a checklist designed for acute bioassays using 30 day old fathead minnows. The checklist was accompanied by use-guidelines and definitions of terminology. Copies are available from this laboratory upon request. It was divided into major categories covering equilibrium, locomotor activities, fish dispersion, body movements and coloration, pathology and unique behaviors not previously defined.

The checklist format was designed to provide a standard procedure for converting observational data into a numerically coded form. Each code number, representing a particular abnormality, was then placed in computer data files for subsequent analyses. With minor exception, each code was based on a mutually exclusive event. For example, the fish could be either lighter (code 81) or darker (code 82) in body color, but not both, compared to controls.

Test Procedures

Observational procedures--Observations were made for several minutes to gain an overview of gross behaviors, then more closely for subtle abnormalities. Lastly, the observor passed a hand over the tanks assessing the overhead fright (startle) response of the fish. If a tank of fish did not respond to this stimulus, the tank was rapped lightly to determine their response to a vibrational stimulus. These procedures were repeated several times daily during the four day test and the data reported for each time interval represented a "summary of events" for that time period.

Video recording/documentation--Video recordings (special test area) were made at each scheduled time interval and a checklist was completed in conjunction with these taping sessions. Video recordings were also made in the regular acute bioassay room whenever unique behaviors occurred. These

raw data tapes were studied for possible behaviors missed by visual observation and are on file at this laboratory.

Data entry and analyses--Behavioral code data were placed in entryprotected computer files [26] according to test concentration and time of observation for each chemical tested. A Digital PDP 11/70 was used for all data entry and subsequent analyses. These entries and any required updates were performed solely by the senior author.

Initial data analyses were conducted using resident computer programs. Percent occurrence of each behavior was determined for individual chemicals. Percentages were calculated on the basis of a matrix having a maximum of 25 cells (5 concentrations x 5 time intervals). However, only cells with live fish were used in order to best represent these data. For example, if all fish died at the highest concentration a 4 x 5 cell matrix (N=20) would be used.

The statistical properties for each of the variables was examined by evaluating the mean, median, skewness and kurtosis. Log transformations were used to stabilize the variance for those variables in which skewness or kurtosis was improved from the original measurements. All statistical calculations [27] were performed using BioMedical Computer Program (BMDP-79) [28].

## RESULTS AND DISCUSSION

The 139 chemicals tested to date have been tabulated in Appendix A according to behavioral/morphological change, mortality pattern and chemical group. Further explanatory information regarding these data is presented in Appendix B. Behavioral changes judged to be innocuous were deleted. Relative trends, expressed as percent occurrence, are based on the data in Appendix A.

### Behavioral Pattern of Controls

Control fish maintained a fairly compact school, covering about 1/3 of the tank bottom, during the first two days of the four day exposure. By the 3rd or 4th day the school tends to become less compact covering up to 2/3 of the tank area. In terms of vertical distribution, they spend most of their time in the lower 2/3 of the tank. When startled, they instantly form a tight school that is maintained for a brief period. They become slightly less responsive, i.e., respond more slowly and form a less dense school, toward the end of the test. Fish were usually moderately active and exhibited no other extraordinary behaviors.

### Non-observed and Contradictory Events

Events not observed--Potential behavior and morphological data were lost in two ways. First, events were not recorded for a pre-scheduled time interval. Loss due to these oversights was minimal. Second, events taking place between observational periods obviously were not recorded. Since frequent observations were made throughout the day and the data represents a 'summary of events' for each given time interval, these losses were not a major problem especially at the lower concentrations.

The main loss of data occurred in higher concentrations with chemicals causing immediate death. Fish appeared unstressed when observed at one hour,

but were dead, or nearly dead, when seen less than an hour later. This might account for unobserved schooling behaviors among all chemicals tested. Expenditure of resources overcoming this problem was not practical, in that 2-4 bioassays were conducted concurrently.

Contradictory events--Some chemicals elicited behaviors seemingly contradictory upon superficial examination of the data. There are cases where the fish were as active as they were inactive; as overreactive as underreactive to outside stimuli; etc. Upon examination of the data in relation to time of occurrence, many of these apparent discrepancies can be explained. It was not uncommon for a narcosis producing chemical to cause fish to become hyperactive early in the exposure as a result of a chemosensory irritant or avoidance response. Conversely, fish in an extremely hyperactive state were so stressed that they simply did not respond to any outside stimuli. Fortunately very few ties in activity were encountered and behaviors that appeared most often, did in fact, represent the overall trend.

Equilibria

Loss of equilibrium [29,30] was initially thought to be a key endpoint for identifying chemicals causing death through narcosis. Most of the chemicals studied (84%) caused fish to lose equilibrium (loss of the righting reflex) in one form or another. Prolonged loss of equilibrium was not necessarily indicative of narcosis. Several chemicals caused severe swelling of the body cavity which forced the fish to swim upside-down for several days.

Observations prior to this study suggest that some chemicals cause fish to 'just die' without any apparent symptoms preceding death. These fish seemed to either die and roll over, or vice-versa. Based on this study, it

appears that fish which die suddently or exhibit very few behavioral symptoms prior to death are being killed through an uncoupler MOA.

Thus, the type and time of equilibrium loss appears to be an important factor. If fish roll over, lay on their sides or hang vertically or are suspended in mid-water, then the loss probably indicates narcosis. These symptoms normally disappear among survivors as the exposure progresses. Loss of equilibrium associated with other symptoms is more indicative of some other MOA.

Locomotor Activities

Spontaneous locomotor activity--Most of the test chemicals (55%) caused narcosis [8] and hypoactivity. The fish either remained inactive throughout the test or began to show signs of recovery toward the end of the test. Occasionally the reduction in overall activity was preceded by a period (0-8 hrs) of increased activity attributable to a transient chemosensory response [31].

A few chemicals caused hypoactivity at nearly all concentrations during the test. The MOA of these chemicals was definitely not narcosis, but rather a severe state of toxicosis resulting in depressed locomotor activity. They also showed other signs of stress such as: edema, hemorrhage, scoliosis and lordosis. Some chemicals of this type included: 4-benzoylpyridine and pentachloropyridine (group 15.3-pyridines), triphenyl phosphate (group 19.0-phosphorous compounds), and carbaryl (group 21.0-carbamates).

Hyperactivity [32] was caused by 32% of the chemicals tested. Presently, it appears that most of these represent uncouplers, AChE inhibitors and a stimulant-like MOA. Such chemicals were predominantly from the following chemical groups: 5.0-aldehydes, 8.3-acrylates, 10.3-primary amines,

13.1-halogenated benzenes, 14.0 and 14.1-phenols, and 21.0-carbamates. The rest of the chemicals (13%) caused no observable change in activity level.

Four of the six sulfur compounds (groups 12.0 and 12.2) caused a mixture of activity and produced a set of symptoms which were different compared to other chemical groups. For example, the fish were either curved to the right or the left, but showed no signs of the typical scoliosis/hemorrhagic areas (see also spiral swimming).

Startle (fright) response--Reactions to visual (overhead fright response) and vibrational (escape response) stimuli were first divided into three categories: overreactive, narcotized and anesthetized. The latter two were combined into 'underreactive' since they represent degree of response and were not useful as separate endpoints to assess MOA.

A majority of the chemicals (58%) caused the fish to become less reactive when subjected to outside stimuli [33]. No observable change in reactivity was found for 23% of the chemicals and the remaining 19% caused the fish to become overreactive. Loss of reactivity was common to narcotic chemicals with the following exception. Fish were less sensitive to outside stimuli when stressed so intensely that they were unable to respond to such signals.

Corkscrew and spiral swimming--Just over half of the chemicals caused fish to swim in a corkscrew and/or spiral pattern. Prior to this study these abnormal swimming behaviors were thought to be more descriptive of non-narcotic chemicals. However, many narcotic chemicals caused fish to swim slowly in a corkscrew fashion due to repeated loss of equilibrium as they swam. A different MOA was indicated when corkscrew swimming was associated with hyperactivity or when fish swam in a spiral manner. Spiral swimming may

require further study in that a narcosis chemical, having a sulfur group in its' structure, may cause tetany resulting in spiral swimming movements.

Fish Dispersion and Social Behaviors

Loss of schooling (horizontal distribution)--On a horizontal plane, 96% of the chemicals cause loss of the fish's natural ability to form and maintain a school [31,34,35,36]. Vertical dispersion merely represented a greater loss of schooling. The remaining chemicals (4%) probably caused a loss of schooling which was not observed. Loss of schooling was the single most sensitive indicator of stress and was usually the first major symptom to appear. The loss often persisted for hours before other signs of stress became evident. However, it was not a good endpoint for separating chemicals into categories according to MOA because it is more of a 'generic' response. A few chemicals caused fish to form extremely compact schools that were located at the outlet end of the exposure tanks. The significance of overschooling needs more study.

Tank location (vertical distribution)--About 51% of the chemicals examined caused fish to hover at or near the water surface, 35% kept them near the bottom, and 11% caused no observed shift in tank location. Vertical distribution was not a good primary endpoint for separating chemicals according to MOA. Chemicals causing increases in ventilation rate/amplitude or in locomotor activity usually forced fish to surface. Narcotic chemicals also caused fish to frequent or remain near the water surface. A nearly equal distribution was not uncommon for many narcosis chemicals. Although vertical distribution was not considered in the development of a predictive model, it may later prove useful as a secondary qualifier.

Agonistic and cannibalistic behaviors--A small percentage (4%) of the chemicals tested caused agonistic behavior in the fish [37]. In most cases the intensity and duration of the response was minimal as shown below:

| Subgroup Number[a] | Chemical Group | Chemical Name | Description |
|---|---|---|---|
| 7.0 | Carboxylic acids | Salicylic acid - NA | Moderate effect, 24-48 hrs |
| 10.5 | Tert. aromatic amines | N,N-Dimethylbenzylamine | Intense head to head biting, chase and nip sequences, 72-96 hrs |
| 14.0 | Phenols, substituted | P-phenoxyphenol | Mild effect, 24-72 hrs |
| 15.3 | Pyridines | 1-Benzylpyridinium-3-sulfonate | Very mild effect, 24 hrs |
| 15.3 | Pyridines | 3-Hydroxy-2-nitro-pyridine | Moderate effect, 0-8 hrs |

[a] Chemical classification from structure activity research program at ERL-D.

N,N-Dimethylbenzylamine was the one to elicit an intense response which may represent a secondary symptom or some unique MOA [38]. Only two chemicals, both from chemical group 8.0-basic esters caused live fish to pick-at or eat the bodies of dead fish. Cannibalism may come about as a result of some secondary mechanism.

Body Movements and Coloration

Fin movements--Increases in the frequency of fin movement was rarely seen. An increase in pectoral fin 'flicker' was the most common movement

observed [8,39]. Such changes were often difficult to observe among an array of other activities. It was not considered a major endpoint in terms of delineating MOA.

Ventilatory patterns--Approximately 60% of the chemicals caused increases in ventilation rate. During the course of these tests it became clear that this mutually exclusive category was much too broad. Narcotic chemicals caused increases in ventilation rate that can be characterized as being rapid and shallow (opercular amplitude very small) and the increases persisted for about 24 hours.

In contrast, uncouplers and stimulants caused marked increases in ventilation rate and amplitude (albeit hyperactivity was directly responsible for some of these increases). Survivors often had elevated ventilation rates [40] that lasted for days, particularly at the higher concentrations. It is proposed that two categories are needed to assess increases in ventilatory rate among fathead minnows used in future tests. Increases would be classed as (A) rapid and shallow, and usually short-lived, or (B) rapid and of large amplitude, and may persist for days.

Increases in cough rate and other abnormal opercular activities (yawns and gasps) were reported for less than 10% of the chemicals studied. Activities occurring intermittently were extremely hard to observe among groups of small fish which seldom remain totally inactive. Tanks equipped with fixed magnifying lens appear useful for making detailed observations of this kind.

Body coloration (pigmentation)--Chemicals of known or suspected narcotic MOA caused dark coloration in the fish [8,41]. Some caused darkening along the lateral line while others caused the entire fish to appear almost black in color. The response was often graded directly to the concentration.

Non-narcotic chemicals also caused dark coloration, but the darkening in these cases was not always blackish; fish often became brown or dark tan in color. Assessment of changes in body pigmentation may require two categories of coloration.

Although body darkening was thought to be a generic stress response, only 55% of the chemicals caused dark coloration. Forty-one percent caused no change in color. The rest (4%) caused fish to become lighter in color. Lack of color change could be real, just not observed, or was too subtle a change to be distinguished by gross examination. A lighter than normal color change remains under study.

Convulsions and tetany--Convulsions appeared in conjunction with tetany or physical deformities (Table 1) in about half of the cases observed. Seventeen percent of the chemicals, from a broad range of chemical groups, caused fish to convulse. Two categories may be required in the future to more appropriately describe the response. One category would be for convulsive movements that seem to occur independently; i.e., they occur anytime. The second category would include convulsive movements that only occur when the fish swims. Locomotor activity accompanied by shaking-shudder-vibration-convulsive movements may be a useful endpoint for MOA investigations.

Although tetany did in fact occur, the present definition of this response proved to be somewhat ambiguous. Paralysis, representing 'permanent tetany' was combined with tetany since there was no valid reason to distinguish between the two. Only two chemicals were initially labeled as causing paralysis. Also, there was too much similarity between tetany (rigid musculature) and scoliosis (curved laterally). This was especially evident

for the sulfur compounds, thiols and disulfides, which caused lateral curvatures.

Body curvatures, caused by sulfur compounds, were more descriptive of tetany in that no skeletal damage or hemorrhagic areas were evident. The latter being characteristic of what is usually thought of as scoliosis. It is not known if these fish would have recovered had the exposures been stopped. Lastly, tetany was included as a body movement which would be appropriate when the response occurs intermittently. If the rigidity persists, it would be considered a deformity providing the musculature does not return to normal when the fish are placed in clean water. Tetany requires additional clarification.

General Pathology

Deformities—Sixteen chemicals caused some type of 'permanent' deformity (Table 1). Of these, six caused scoliosis only, four caused lordosis only, while the remaining six caused a combination of effects [42,43,44]. The quaternary ammonium compounds produced an obvious 'kink' in the tail, which resembled a check-mark, posterior to the dorsal fin. No convulsions or tetany were observed prior to the appearance of this tail-kink. Virtually all of these chemicals were judged to be AChE inhibitors based on the overall pattern of response and pattern of known inhibitors.

Early in this study it appeared that the deformities were part of a pattern of symptoms that progressed over time as follows: convulsions ⟶ tetany ⟶ hemorrhagic areas ⟶ deformities. It was concluded that deformities are most likely to be preceded by convulsions and tetany. However, the appearance of convulsions or tetany is not always followed by deformities.

Edema--Twenty-one percent of the chemicals caused edema in the abdominal area. The degree of edema was usually mild and often disappeared between 24 and 48 hours. Some anesthetics are known to produce swelling of tissues. Of the 21%, about half of the cases came from chemical groups 5.0-aldehydes and 8.3-acrylates. Several chemicals from these groups caused such severe edema that the abdomens of some fish split open prior to death. Cursory examination revealed dissolution of the entire digestive tract into a water-mush, filling the body cavity. Both edema and protruded eyeballs (popeye) were evident among fish exposed to substituted phenols.

Hemorrhage--A few (8%) of the chemicals caused hemorrhaging. These areas of hemorrhage ranged from one or two large spots to what might be more accurately described as petechia. Benzaldehyde, a purported membrane irritant, caused petechial symptoms which disappeared after 72 hours. Super-hyperactive fish often had snouts which were hemorrhagic, presumedly from hitting the walls of the tanks. Hemorrhagic areas were also observed in the gill region or at the base of the dorsal and pectoral fins. The most common form of hemorrhage occurred along the backbone, particularly in the caudal region. Location was considered important where these data are used to assess MOA.

Backbone area hemorrhage [45] was closely associated with one or more of the following: convulsions (spasms), tetany, scoliosis or lordosis. A combination of these effects was most characteristic of AChE inhibitors. One fish was observed to have a sudden spasm which bent the fish at a right angle for several seconds. It was assumed that such violent contractions damaged or fractured the vertebral column resulting in hemorrhage and some type of permanent deformity. Convulsive, spasmodic movements often appeared as a

near blur to the naked eye. Slow motion playback of video tapes did not help. High speed video imaging will be required for an understanding of the relationship between convulsive behaviors and changes in body morphology.

Miscellaneous--Other abnormalities included abnormal gills - greatly reddened or pale in color; and possible loss of mucous. It was concluded that these are important MOA endpoints but ones that must be viewed using a magnifying lens for proper observation. The present data base lacks necessary detail. Last, greatly elongated anal casts often were observed stringing from fish exposed to narcotic chemicals such as MS-222.

Mortality Pattern

The pattern of fish deaths [46] during the 96 hr exposures was examined. Some chemicals caused death early in the exposure while others caused death throughout the tests. This had more to do with type of chemical rather than concentration level. An LC50 ratio: A + B/2C, where A = 24 hr LC50, B = 48 hr LC50, and C = 96 hr LC50, was used to reflect these variations. The ratio was always close to 1.00 for narcotic chemicals. A ratio of less than 1.50 was calculated for about three quarters of the chemicals tested. The LC50 ratio for the remaining chemicals was either zero or exceeded 1.50. This variable was disregarded for now because: (1) it was decided not to use these data in a predictive model as the variable mandates a 96 hr exposure, thus eliminating the option of making predictions using a shorter time-frame, and (2) inclusion of these data is not essential in deference to other selected behavioral and morphological changes. This does not mean the pattern is unimportant.

MOA of a Binary Mixture

As an aside to this study, the opportunity arose to observe the behaviors of fatheads exposed to a binary mixture. Fish were exposed to

various mixtures (40 1 L tanks containing 10 fish each) of 1-octanol and 2-chloroethanol using a blind-study approach. The latter chemical was earlier found to be 30x more toxic than predicted using a narcosis model [19]. Based on behavioral and morphological change, the various groups of fish (tanks) were rather easily separated into three distinct categories.

Category I included those fish that showed behaviors consistent with narcosis. It was later verified that these fish were exposed to high concentrations of 1-octanol. Category II fish exhibited a pattern of behaviors which were not consistent with narcosis. These proved to be tanks containing high concentrations of 2-chloroethanol. It was also possible to rank the various exposure tanks (Categories I and II only) according to relative concentration based solely on the intensity of behavioral and morphological change, and pattern of death.

Category III fish showed little or no change in behavior as they were exposed to relatively low concentrations of these two chemicals. This approach shows promise for assessing the MOA of complex mixtures [47]. Such insight would help pinpoint the type of chemical(s) which appear to be most biologically active.

Future Endpoints

Several behaviors and morphological changes were observed but not anticipated or overlooked in the preparation of the checklist. The following terms merit consideration with respect to MOA.

Anguiliform Swimming - eel-like swimming; flacid body musculature.

Gasping - resembles a slow cough or fast yawn; does not occur at the air-water interface; should not be confused with gulping.

Gulping - intake of surface water and air while swimming; mouth open and labored ventilation evident.

Nystagmus - rapid eye twitching either forward or backward or side-to-side (may be characteristic of barbiturates).

Pretechia - small reddish spots scattered over the body surface due to intradermal or submucous hemorrhage.

MOA Classification Using Behavioral Indices

Three of the four physiology-type categories, anesthetics (representing narcosis), AChE inhibitors and uncouplers appear useful for inclusion in the behavioral/morphological classification scheme. The only exception involves membrane irritants. Of the two purported irritants, benzaldehyde appeared to act as a mild AChE inhibitor. Acrolein acted more like an uncoupler.

The first step in chemical classification according to MOA was elimination of those variables which were repetitious, of minor biological significance or were supported by a temuous data base. Variables such as jumping and uninterrupted swimming were deleted since they reflect hyperactivity; tail position (body angle) was of little importance, and data involving mucous production were eliminated.

After examining and hand-clustering all remaining data, 14 variables were selected for further analyses. They were (in no particular order): hyperactivity, hypoactivity, corkscrew swimming, spiral swimming, overreactive, underreactive, increase in ventilation, convulsive, tetany, darkening of body coloration, edema, hemorrhagic, scoliosis and lordosis.

Using "known" MOA chemical data and results of the hand-clustering process as guides, chemicals tested thus far fell into one of four categories. These four, based on probable cause of action, were as follows: narcosis, AChE inhibition, uncouplers and stimulant-like. The latter category was assigned because (1) the pattern of response resembled that caused by uncouplers but the fish seemed super-hyperactive, and (2) the

response paralleled that of fatheads exposed (special test) to caffeine, a known stimulant.

Ten different chemicals were selected to represent each of these four categories (40 chemicals total). Selection was based on those chemicals which were most representative of a given category. Characteristic patterns of response for each of these four categories are outlined in Table 2.

Discriminant function analyses (Table 3) revealed that behavioral data could be used to identify and separate chemicals into various groups (Figure 1). Narcosis-acting and AChE inhibitory chemicals fell into clearly defined groups which were distinct from those classed as uncouplers and stimulant-like. The latter two groups were nearly identical in pattern and greatly overlapped as shown in Figure 1. The third discriminant function (axis) is the primary discriminant for groups 2 and 4. It should be possible to classify the remaining 99 chemicals according to probable MOA once a computer-based model is developed for this purpose.

The degree of separation varied when discriminant function analyses were applied separately to 8, 24, 48, 72, and 96 hr data (Table 3). Very poor correlations were obtained when only 8 and 96 hr data were used. The 24 and 48 hr data yielded the best correlation. It appears that data from this time-frame could be used to shorten the test where early predictions are required.

Behavioral data at test concentrations just above and below the calculated 96 hr LC50 values were examined to determine if the response at higher concentrations was markedly different from the overall response (Table 3). In theory, effects at lower concentrations may minimize the gross response. This approach also produced a poor correlation and is not recommended as too much data is eliminated from the analysis.

## SUMMARY AND CONCLUSIONS

1. Although somewhat small in size, 30 day old fathead minnows were found to be a good species for behavioral toxicology studies. They exhibit a wide range of behaviors and morphological changes in response to chemicals of diverse types. They also are a proven species for conducting acute and chronic bioassays.

2. Loss of schooling behavior was the most sensitive stress indicator. It was considered a "generic response" as virtually all chemicals considered caused loss of the fish's ability to form and maintain a fish school.

3. Fathead minnows exhibited a number of unique behaviors which, when studied further, may help delineate minor modes of action within a major MOA category.

4. Other behaviors may prove useful in the development of a model for predicting MOA. Some of these potential endpoints are so subtle that magnification will be required to properly monitor these aberrations.

5. It was concluded that a model using selected behaviors and morphological change shows promise for predicting the most likely MOA of an unknown chemical compound or a complex mixture. When these data are included with physiological, biochemical, and histological changes it may be possible to develop a much more sophisticated model.

ACKNOWLEDGMENTS

We thank C. Stidley and P. Pollock for development and modification of resident data management programs. Special thanks goes to Dr. G. Niemi for his advice concerning multivariate analyses. We also appreciate the efforts of ERL-D Toxic Research Branch members and University of Wisconsin Center for Environmental Studies personnel for conducting acute bioassays and performing physical-chemical analyses.

REFERENCES


[1] Warner, R.E., Bull. World Health Org., Vol. 36, 1967, pp. 181-207.

[2] Oakley, R.S., Drugs, Society and Human Behavior, C.V. Mosby Co., St. Louis, 1972, p. 50.

[3] Rand, G.M. in Fundamentals of Toxicology, Hemisphere Publ. Corp., New York, 1985, Chapter 9, pp. 221-263.

[4] Irwin, S. in Animal and Clinical Pharmacologic Techniques, Yearbook Medical Publ., Chicago, 1964, pp. 36-54.

[5] Warner, R.E. and Peterson, K.K., J. Appl. Ecol., Vol. 3 (Suppl.), 1966, pp. 223-247.

[6] Matthews, J.E. and Myers, L.H., Pathological Effects of Chemicals on Aquatic Organisms, Workshop, U.S. Envir. Prot. Agency, Ada, OK. May 8-9, 1973.

[7] Tilson, H.A. and Mitchell, C.L., Ann. Rev. Pharmacol. Toxicol., Vol. 24, 1984, pp. 425-450.

[8] McFarland, W.N., Publ. Inst. Mar. Sci., Vol. 6, 1959, pp. 23-55.

[9] Schoettger, R.A. and Julin, A.M., Invest. in Fish Control, U.S. Dept. Interior, Rept. No. 13, 1967.

[10] Goldstein, D.B., Ann. Rev. Pharmacol. Toxicol., Vol. 24, 1984, pp. 43-64.

[11] Ahlborg, U.G. and Thunberg, T.M., CRC Critical Rev. in Toxicol., July 1980, p. 16.

[12] Cleveland, L., et al., Envir. Toxicol. Chem., Vol. 1, 1982, pp. 205-212.

[13] Basol, M.S., et al., J. Envir. S. B., Vol. 15, 1980, pp. 993-1004.

[14] Mount, M.E. and Oehme, F.W., Residue Reviews, Vol. 80, 1981, pp. 1-64.

[15] Barrow, C.S., CIIT Activities, Vol. 2, Sept. 1982, pp. 3-5.

[16] Reiter, L.W. [Ed.], "Behavioral toxicology an emerging discipline", Tech. Report PB291841, Natl. Tech. Info. Ser., Springfield, VA, Dec. 1977.

[17] Brungs, W.A. and Mount, D.I., "Introduction to a discussion of the use of aquatic toxicity tests for evaluation of the effects of toxic substances", Tech. Publ. 657, ASTM, Philadelphia, PA, 1978, pp. 15-26.

[18] Reynolds, W.W. and Casterlin, M.E. in Biological Monitoring of Fish, Lexington Books, Lexington, MA, Chapter 4, pp. 59-82.

[19] Veith, G.D., et al., Can. J. Fish. Aquat. Sci., Vol. 40, 1983, pp. 743-748.

[20] Benoit, D.A., et al., Water Res., Vol. 16, 1982, pp. 457-464.

[21] Brooke, L.T., et al., "Acute toxicities of organic chemicals to fathead minnows", Center for Lake Superior Envir. Studies, Univ. of Wis., Superior, Vol. 1, 1984.

[22] Olla, B.L. [Ed.] in Marine Bioassays, Marine Tech. Soc., Wash., D.C., Workshop Proceedings, 1974, pp. 1-31.

[23] Fentress, J.C. in Perspectives in Ethology, Plenum Press, New York, Vol. 1, 1973, p. 158.

[24] Meyers, L.S. and Grossen, N.E., Behavioral Research, 2nd ed., W.H. Freeman and Co., San Francisco, 1978.

[25] Marcucella, H. and Abramson, C.I. in The behavior of fish and other aquatic animals, Academic Press, New York, Chapter 2, 1978, pp. 33-77.

[26] Cairns, Jr., J. and Van Der Schalie, W.H., Wat. Res., Vol. 14, 1980, pp. 1179-1196.

[27] Aspey, W.P. and Blankenship, J.E. in Quantitative Methods in the Study of Animal Behavior, Academic Press, New York, 1977, pp. 75-120.

[28] Dixon, W.J. and Brown, M.B. [Eds.], "BMDP-79 Biomedical Computer Programs P-Series", Univ. Calif. Press, Berkeley, 1979.

[29] Bengtsson, B.E., et al., Chemosphere, Vol. 13, 1984, p. 616.

[30] Holcombe, G.W., et al., Envir. Poll. (Series A), Vol. 35, 1984, pp. 367-381.

[31] Brown, S.B., et al. in Chemoreception in Fishes, T.J. Hara, Ed., Elsevier Sci. Publ. Co., New York, 1982, Chapter 20, pp. 363-393.

[32] Weis, P. and Weis, J.S., Envir. Res., Vol. 7, 1974, pp. 68-74.

[33] Eaton, R.C. and Hackett, J.T, in Neural Mechanisms of Startle Behavior, Plenum, New York, 1984, Chapter 8, pp. 213-266.

[34] Johnson, F.N., Pharmacopsychiat, Vol. 14, 1981, pp. 208-212.

[35] Burgess, J.W., Pharmacol. Biochem. and Behav., Vol. 17, 1982, pp. 137-140.

[36] Holcombe, G.W., et al., Envir. Poll. (Series A), Vol. 29, 1982, pp. 167-178.

[37] Kwasnik, J.F., "Effects of cadmium on the predator-prey behavior of fathead minnows and largemouth bass in a model ecosystem", Ph.D. Thesis, Purdue Univ., August 1977.

[38] Keller, D.L. and Umbriet, W.W., Science, Vol. 124, 1957, p. 407.

[39] Henry, M.G. and Atchison, G.J., Envir. Biol. Fish., Vol. 4, 1979, pp. 27-32.

[40] Carlson, R.W., Envir. Poll. (Series A), Vol. 29, 1982, pp. 35-56.

[41] Scott, G.T. in Pigmentation: its genesis and biological control, V. Riley, Ed., Appleton-Century-Crafts, New York, 1972, Chapter 26, pp. 327-342.

[42] Mayer, F.L., et al., "Collagen metabolism in fish exposed to organic chemicals", Ecol. Res. Ser. EPA-600/3-77-085, Natl. Tech. Info. Ser., Springfield, VA, 1977.

[43] Bengtsson, B.E., Phil. Trans. R. Soc. Lond. B., Vol. 286, 1979, pp. 457-464.

[44] Kumar, K. and Ansari, B.A., Pestic. Sci., Vol. 15, 1984, pp. 107-111.

[45] McCann, J.A. and Jasper, R.L., Trans. Am. Fish. Soc., Vol. 101, 1972, pp. 317-322.

[46] Smock, L.A., et al., Wat. Res., Vol. 10, 1976, pp. 537-543.

[47] Ellis, D.V., Mar. Poll. Bull., Vol. 15, 1984, pp. 163-164.

Figure 1. -- BMDP discriminant function analysis plot of 40 chemicals clustered according to four probable modes of action: 1 = narcosis; 2 = uncouplers; 3 = AChE inhibitors; 4 = stimulant-like. Circled numbers show group mean. * = overlap of different groups.

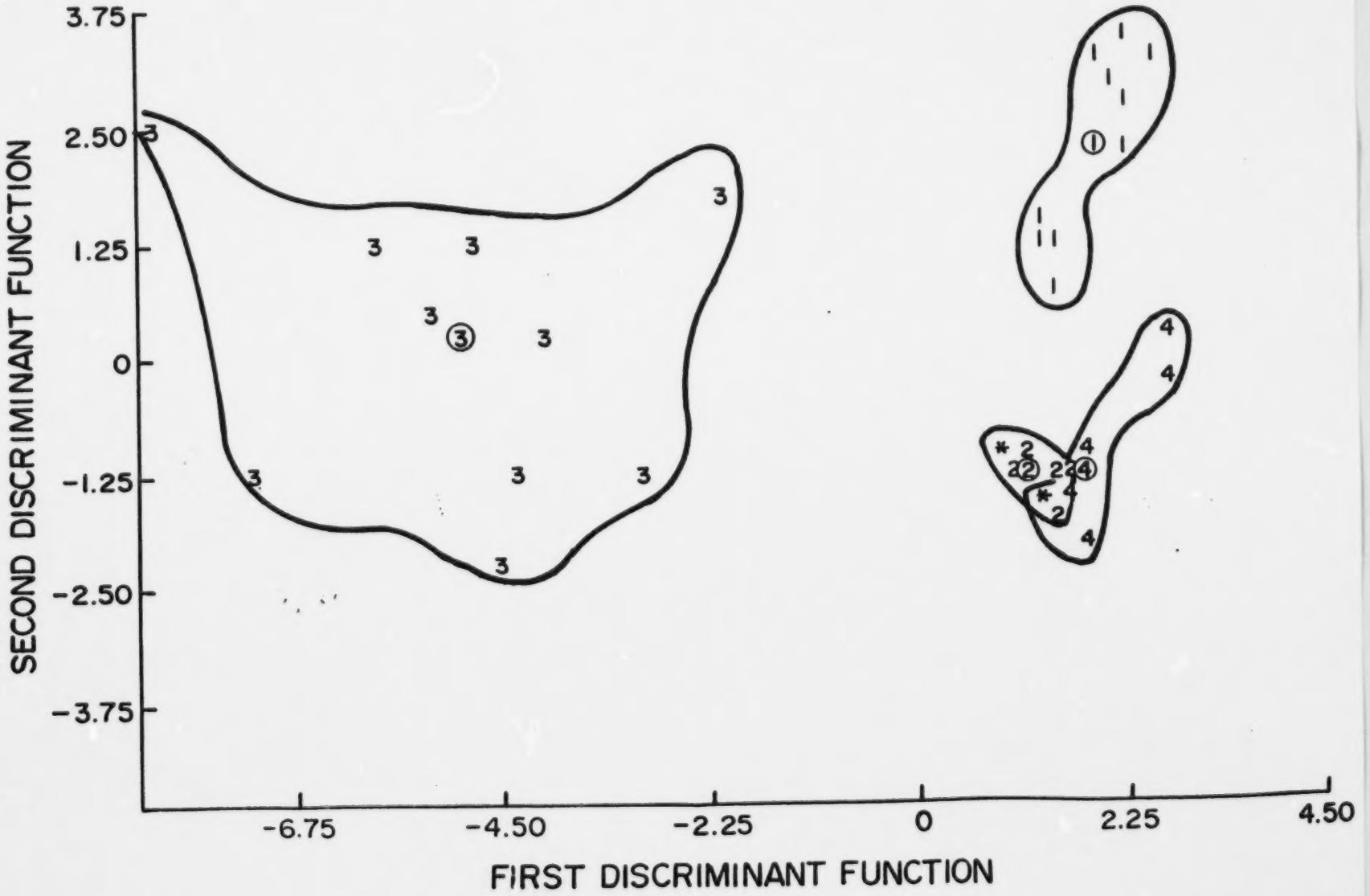

Table 1. — Chemicals causing physical deformities among 30 day old fathead minnows (96 hr exposure).

| Chemical Group | Number of Chemicals Causing: Scoliosis Only | Lordosis Only | Combination |
|---|---|---|---|
| 3.1 - Diphenylethers | 1 | 0 | 0 (3)[b] |
| 5.0 - Aldehydes[a] | 0 | 0 | 1 (13) |
| 8.3 - Acrylates[a] | 1 | 0 | 1 (11) |
| 15.3 - Pyridines[a] | 2 | 1 | 2 (16) |
| 15.4 - Triazines | 1 | 0 | 0 (1) |
| 19.0 - Phosphorus cmpds.[a] | 1 | 0 | 0 (4) |
| 20.0 - Quat. ammon. cmpds. | 0 | 3 | 0 (6) |
| 21.0 - Carbamates[a] | 0 | 0 | 2 (4) |

[a] Convulsions or tetany evident prior to appearance of a physical deformity.

[b] Total numbers of chemicals tested for each chemical group shown in parentheses.

Table 2. -- Behavioral and morphological events which characterize the four selected categories describing mode of action.

| Behavioral/Morphological Event | Most Typical Pattern of Response | | | |
|---|---|---|---|---|
| | Narcosis | Uncoupler | AChE Inhibitor | Stimulant-Like |
| Loss of Equilibrium: | Fish lay on sides or are suspended vertically, often for long periods of time preceding death. | Loss of equilibrium usually occurs just prior to death. | Loss of equilibrium associated with other symptoms of stress. | Loss of equilibrium associated with other symptoms of stress. |
| Spontaneous Swimming Activity: | Swimming movements showed hypoactive. May be slightly hyperactive during initial exposure. | Hyperactive. | Tend to be hypoactive. Some chemicals caused hyperactivity | Hyper- to super-hyperactive. May exhibit agonistic behavior. Jumping and burst of swimming common. |
| Corkscrew/Spiral Swimming: | Minor corkscrew swimming evident; associated with fishes' attempt to maintain equilibria. | May exhibit spiral swimming movements. | High incidence of both corkscrew and spiral swimming for extended periods of time. | Minor incidence of corkscrew swimming. |
| Startle Response: | Reaction to outside stimuli greatly reduced or non-existent. May be slightly overreactive during initial exposure. | Tend to be overreactive. | Usually overreactive to outside stimuli. | May be over or underreactive to outside stimuli - depends on level of stress[a]. |
| Ventilatory Pattern: | May show a rapid and shallow increase in opercular movements early in the exposure. | Increase in opercular rate; amplitude of movements large. | Increase in opercular rate; amplitude of movements large. | Increase in opercular rate; amplitude of movements large. |
| Body Movements: | No gross changes in body movement. | No gross changes in body movement. | May show signs of tetany and convulsive movements. | Tails may "vibrate" during swimming. |
| Body Coloration: | Usually very dark (blackish) in color. Response often graded to the concentration. | May or may not be dark (brownish) in color. | Dark in coloration. | May be dark or brownish in color. |
| Hemorrhage: | No hemorrhage. | Hemorrhage not usually evident. | Hemorrhagic areas present, particularly in caudal area. | Snouts may show signs of hemorrhage. |
| Deformities: | May exhibit signs of mild edema (swollen bellies) early in the exposure. | May exhibit signs of mild edema. | High incidence of scoliosis and/or lordosis. Could exhibit signs of severe edema. | Could exhibit signs of severe edema. |
| Mortality Pattern: | Most fish die in less than 48 hr, usually 24. | Most fish die 24-72 hrs. | Most fish die 24-96 hrs. | Fish die throughout the test. |
| Chief Characteristics: | Hypoactive, underreactive to outside stimuli, very dark (blackish) in color, respiration rapid and shallow. | Fish are hyperactive and overreactive. Most fish just die without exhibiting numerous signs of stress. | Generally hypoactive but overreactive to stimuli; high incidence of abnormal swimming movements and deformities. Exhibit numerous signs of stress. | Fish extremely hyperactive 0-96 hr; exhibit agonistic or other unusual behaviors, deaths occur at random during the exposure. |

[a] Highly stressed (hyperactive) fish tend not to respond to outside stimuli.

Table 3. -- Percentages of cases correctly classified using BMDP discriminant function analysis. N = 40 chemicals.

| Classification Category | 96 hr Exposure | Time Interval Only (hr) | | | | | LC50 Bracket[a] |
|---|---|---|---|---|---|---|---|
| | | 8 | 24 | 48 | 72 | 96 | |
| (1) Narcosis | 100 | 90 | 90 | 90 | 90 | 100 | 88.9 |
| (2) Uncoupler | 80 | 0 | 70 | 90 | 80 | 10 | 88.9 |
| (3) AChE Inhibitor | 100 | 0 | 90 | 90 | 90 | 80 | 100.0 |
| (4) Stimulant-like | 80 | 100 | 80 | 90 | 60 | 80 | 44.4 |

[a] Behavioral data from concentrations immediately above and below the 96 hr LC50 value.

Appendix A. -- Numbers of chemicals tested according to the most prevalent behavioral/morphological response, mortality pattern[a], and chemical group[b].

| | Chemical Groups | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2.1[c] | 3.0 | 3.1 | 4.0 | 5.0 | 6.0 | 6.1 | 7.0 | 8.0 | 8.1 | 8.2 | 8.3 | 9.0 | 10.0 | 10.1 | 10.2 | 10.3 | 10.5 | 12.0 | 12.2 | 13.1 | 14.0 | 14.1 | 15.0 | 15.2 | 15.3 | 15.4 | 15.5 | 15.6 | 16.0 | 18.0 | 19.0 | 20.0 | 21.0 | Grand Totals |
| No. Chemicals Tested: | 1 | 1 | 3 | 3 | 13 | 1 | 2 | 1 | 7 | 3 | 4 | 11 | 1 | 3 | 3 | 7 | 10 | 5 | 5 | 1 | 3 | 2 | 5 | 4 | 1 | 16 | 1 | 2 | 3 | 2 | 1 | 4 | 6 | 4 | 139 |
| EQUILIBRIA | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Loss w/stress: | 1 | 1 | 3 | 3 | 8 | 1 | 2 | 1 | 7 | 3 | 4 | 11 | 1 | 2 | 1 | 6 | 9 | 3 | 5 | 1 | 2 | 2 | 4 | 3 | 1 | 16 | 1 | 2 | 2 | 2[g] | 1 | 4 | 2 | 4 | 117[c] |
| Loss w/o stress: | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1[c] |
| LOCOMOTOR ACTIVITIES | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Increased activity: | 1 | 0 | 2 | 0 | 6 | 0 | 1 | 1 | 0 | 0 | 0 | 6 | 0 | 1 | 0 | 1 | 4 | 0 | 2 | 0 | 3 | 1 | 3 | 2 | 0 | 1 | 0 | 0 | 3 | 0 | 0 | 2 | 1 | 3 | 44[c] |
| Decreased activity: | 0 | 1 | 1 | 3 | 4[g] | 1 | 1 | 0 | 6 | 2 | 4 | 5 | 1 | 2 | 2 | 6 | 4 | 4 | 5 | 1 | 0 | 0 | 0 | 0 | 1 | 10[g] | 1 | 2 | 0 | 2 | 1 | 5[g] | 4 | 1 | 76[c] |
| Overreactive: | 0 | 0 | 1 | 0 | 3 | 1 | 1 | 1 | 0 | 0 | 0 | 5 | 0 | 1 | 0 | 0 | 3 | 0 | 0 | 0 | 2 | 1 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 2 | 27[c] |
| Underreactive: | 0 | 1 | 2 | 2 | 8 | 2 | 1 | 0 | 5 | 3 | 4 | 5[g] | 0 | 2 | 2 | 6 | 7 | 5[g] | 5 | 1 | 1 | 1 | 1 | 3 | 1 | 5 | 0 | 0 | 0 | 0 | 0 | 3 | 4 | 2 | 80[c] |
| Corkscrew swimming: | 0 | 0 | 2 | 1 | 4 | 1 | 1 | 0 | 1 | 1 | 1 | 4 | 0 | 0 | 0 | 0 | 2 | 1 | 4 | 1 | 2 | 2 | 1 | 0 | 1 | 6 | 0 | 0 | 0 | 1 | 1 | 2 | 1 | 3 | 43 |
| Spiral swimming: | 0 | 0 | 0 | 0 | 6 | 1 | 0 | 0 | 0 | 0 | 1 | 2 | 0 | 0 | 0 | 1 | 2 | 0 | 4 | 0 | 1 | 1 | 1 | 0 | 1 | 6 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 3 | 39 |
| SCHOOLING/SOCIAL BEHAVIOR | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Loss of schooling: | 1 | 1 | 3 | 3 | 13 | 1 | 2 | 1 | 6[g] | 3 | 4 | 11 | 1 | 3 | 2 | 5 | 10 | 5 | 5[g] | 1 | 3 | 2 | 5 | 4 | 1 | 16 | 1 | 2 | 3 | 2 | 1 | 3 | 6 | 4 | 134[c] |
| Formed dense schools: | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 4[c] |
| Located at surface: | 1 | 1 | 2 | 0 | 10 | 0 | 2 | 0 | 5 | 2 | 0 | 10 | 0 | 1 | 1 | 2 | 4 | 3 | 1 | 1 | 2 | 0 | 5 | 3 | 0 | 10 | 1 | 2 | 2 | 2 | 0 | 0 | 2 | 3 | 76[c] |
| Located at bottom: | 0 | 0 | 1 | 5 | 2 | 1 | 0 | 1 | 5 | 0 | 3 | 1 | 1 | 2 | 0 | 6 | 4 | 2 | 3 | 0 | 1 | 2 | 0 | 0 | 1 | 5 | 0 | 0 | 0 | 0 | 1 | 4 | 0 | 1 | 48[c] |
| Agonistic behavior: | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 6 |
| Cannibalistic behavior: | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 |
| BODY MOVEMENT/COLORATION | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Increased fin movement: | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 6 |
| Convulsive: | 0 | 1 | 0 | 0 | 2 | 0 | 1 | 0 | 1 | 0 | 0 | 2 | 0 | 0 | 0 | 2 | 0 | 0 | 1 | 0 | 2 | 1 | 0 | 0 | 0 | 7 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 23 |
| Tetany: | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 1 | 0 | 3 | 1 | 2 | 0 | 0 | 0 | 1 | 2 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 1 | 17 |
| Light in color: | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 5[c] |
| Dark in color: | 1 | 1 | 1 | 3 | 6 | 0 | 1 | 0 | 3 | 1 | 4 | 4 | 0 | 2 | 1 | 4 | 7 | 3 | 5 | 1 | 2 | 0 | 1 | 2 | 1 | 10 | 0 | 2 | 1 | 1 | 0 | 3 | 2 | 4 | 71[c] |
| VENTILATORY PATTERNS | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Increased ventilatory rate: | 1 | 0 | 4 | 1 | 6 | 0 | 2 | 0 | 6 | 2 | 2 | 11 | 1 | 0 | 0 | 1 | 5 | 3 | 5 | 1 | 1 | 1 | 5 | 1 | 0 | 9 | 1 | 0 | 1 | 1 | 1 | 3 | 5 | 4 | 80 |
| Increased cough rate: | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 12 |
| Abnormal opercular activity: | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 7 |
| GENERAL PATHOLOGY | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Scoliosis: | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 2 | 12 |
| Lordosis: | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 3 | 2 | 1 | 11 |
| Edema: | 0 | 0 | 2 | 0 | 6 | 1 | 1 | 0 | 0 | 0 | 0 | 8 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 5 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 2 | 29 |
| Hemorrhagic: | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1[d] | 0 | 1 | 0 | 0 | 1[e] | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 2 | 11 |
| Abnormal gills: | 0 | 0 | 0 | 0 | 2 | 0 | 1 | 0 | 1 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 3 | 0 | 1 | 0 | 2 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 3 | 16 |
| MORTALITY PATTERN[b] | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| LC50 ratio = 0: | 0 | 0 | 0 | 0 | 4[e] | 0 | 1 | 0 | 1[f] | 0 | 0 | 3 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 2 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 18 |
| LC50 ratio < 1.50: | 1 | 1 | 2 | 3 | 6 | 1 | 1 | 1 | 5 | 3 | 3 | 2 | 1 | 2 | 2 | 7 | 8 | 5 | 3 | 0 | 1 | 1 | 4 | 3 | 1 | 15 | 0 | 2 | 2 | 2 | 1 | 4 | 5 | 2 | 100 |
| LC50 ratio $\geq$ 1.50: | 0 | 0 | 1 | 0 | 3 | 0 | 0 | 0 | 1 | 0 | 1 | 6 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 21 |
| Total No. Chemicals/Group: | 1 | | 4 | 3 | 13 | | 3 | 1 | | | | 25 | 1 | | | | | 28 | | 6 | 3 | | 7 | | | | | | 27 | 2 | 1 | 4 | 6 | 4 | 139 |

NOTE: See Appendix B for footnotes and index to chemical groups.



Reproduced from
best available copy.

Appendix B. -- Index to chemical groups and footnotes presented in Appendix A.

| Chemical Class | Sub-Group Number[b] | Description |
|---|---|---|
| (2) Halogenated aliphatic hydrocarbons | 2.1 | Un-saturated |
| (3) Ethers | 3.0 | Basic |
| | 3.1 | Diphenyl |
| (4) Alcohols | 4.0 | Basic |
| (5) Aldehydes | 5.0 | All |
| (6) Ketones | 6.0 | Basic |
| | 6.1 | β-diketones |
| (7) Carboxylic acids | 7.0 | All |
| (8) Esters | 8.0 | Basic |
| | 8.1 | Phthalates |
| | 8.2 | Amides |
| | 8.3 | Acrylates |
| (9) Nitriles | 9.0 | All |
| (10) Amines | 10.0 | Primary aliphatic |
| | 10.1 | Secondary aliphatic |
| | 10.2 | Tertiary aliphatic |
| | 10.3 | Primary aromatic |
| | 10.5 | Tertiary aromatic |
| (12) Sulfur compounds | 12.0 | Thiols |
| | 12.2 | Disulfides |
| (13) Benzenes | 13.1 | Halogenated |
| (14) Phenols | 14.0 | All substituted |
| | 14.1 | Halogenated |
| (15) Heterocyclic nitrogen compounds | 15.0 | Piperazines |
| | 15.2 | Pyrimidines |
| | 15.3 | Pyridines |
| | 15.4 | Triazines |
| | 15.5 | 5-membered ring aliphatic |
| | 15.6 | 5-membered ring aromatic |

Appendix B. -- (continued)

| Chemical Class | Sub-Group Number | Description |
|---|---|---|
| (16) Compounds with two or more hetero atoms | 16.0 | All |
| (18) Anilides and urea's | 18.0 | All |
| (19) Phosphorus compounds | 19.0 | All |
| (20) Quaternary ammonium | 20.0 | All |
| (21) Carbamates | 21.0 | All |

Footnotes:

[a] Mortality pattern based on LC50 Ratio = $\frac{A + B}{2C}$ where A = 24 hr LC50, B = 48 hr LC50, and C = 96 hr LC50.

[b] Chemical classification from structure activity research program at ERL-D. Missing sub-group numbers (10.4 e.g.) indicates no behavioral data recorded to date.

[c] Number of chemicals entered on the basis of whichever response occurred most frequently (e.g., increase or decrease).

[d] Hemorrhagic snouts only.

[e] Chemistry problems for 2 of the 4 chemicals.

[f] Calculated using the 48 hr LC50.

[g] Includes one tied value.